REMARKS

Claims 1, 12, 13, 16-17, and 33-38 are currently pending in the application. By this amendment, claims 1, 12, 13 and 16 are amended, claims 2, 14-15 and 31-32 are cancelled (being drawn in part to a non-elected invention; claims 3-11 and 18-30 were previously cancelled) and new claims 35-38 are added for the Examiner's consideration. The foregoing separate sheets marked as "Listing of Claims" shows all the claims in the application, with an indication of the current status of each.

Claim Rejections: 35 USC § 112, second paragraph

Claims 16-17 and 33-34 stand rejected under 35 USC § 112, second paragraph, as indefinite.

Claims 16-17 and 33-34 depend from claims 12 and 13, respectively. Amendments to claims 12 and 13 have been made to reflect the response to the Restriction Requirement, i.e. these 2 independent claims now recite that a catechin or an antibody is screened by the method of the invention. The recitation of catechin in claims 12 and 13 renders claims 16-17 and 33-34 definite, since they refer back to the catechin.

In addition, Claims 16 and 33 are hereby amended to recite that the subject catechin has a galloyl group, thereby further clarifying the subject matter. Support for this amendment is found, for example, in paragraph [0072] of the published application.

In view of the foregoing, Applicant respectfully requests reconsideration and withdrawal of this rejection.

Claim Rejections: 35 USC § 112, first paragraph

Claims 1-2, 12-17, and 31-34 stand rejected under 35 USC § 112, first paragraph, due to a purported lack of enablement. This rejection is traversed.

Claims 2, 14-15 and 313-32 have hereby been cancelled, thereby making moot this portion of the rejection.

Examiner's rejection of these claims is based largely on the argument that *in vitro* testing cannot be extrapolated to *in vivo* therapy results, stating that there are many cases in which the *in vitro* anti-cancer effect does not match with the *in vivo* anti-cancer effect.

However, as Hou et al., 2004 points out, the anticancer effect of catechins including EGCG have previously been tested with concentrations that are significantly higher than physiological concentrations. In contrast, the concentrations of EGCG used in the development of the anti-cancer assays of the present invention were much lower physiological concentrations (0.1 mM and 1 mM). Employing these concentrations, the inventor identified the 67 kDa laminin receptor as a molecule which is essential for EGCG to exhibit an effect on or inhibit cancer cell proliferation.

Based on this finding, it has already been proven by *in vivo* testing that the anti-cancer effect of EGCG can be shown against cancer cells expressing the 67 kDa laminin receptor. (See attached publication to Umeda et al., *Journal of Biological Chemistry*, 2008, 283: 3050-3058). Considering this finding, the *in vitro* effect can be extrapolated to the *in vivo* effect in this case. That is, one skilled in the art can expect, based on positive *in vitro* screening results obtained with a compound using the 67 kDa laminin receptor according to the practice of the present invention, that the compound will display anti-cancer effects *in vivo*.

In addition, Examiner's rejection of these claims is based largely on the argument that *in vitro* testing cannot be extrapolated to *in vivo* therapy results, even though, as explained above, in the present case such an extrapolation was correct. Applicant respectfully notes that the claims of the invention are not directed to methods of cancer therapy. Rather, they are directed to methods of screening compounds that may have a cell growth-inhibiting effect or a cancer cell metastasis activity-inhibiting effect. While such screening methods are not necessarily definitive, they are a crucial first step in the identification of therapeutic compounds, and the vetting of potential drugs is necessary to eliminate those that are unlikely to work, and useful to identify those that show promise for further investigation.

Claims 1, 12 and 13 have hereby been amended to clarify that the subject matter of these claims is a method of screening test compounds that *may have* the effect of inhibiting cell growth or cancer cell metastasis activity. Support for this amendment is found in paragraph [0113] of

published application. The screening is based on assessing a test compound's ability to bind to 67 kDa, which is a reasonable first step in such a screening process since 67 kDa has been implicated in cell growth and cancer cell metastasis.

In view of the foregoing, Applicant respectfully requests reconsideration and withdrawal of this rejection.

Claim Rejections: 35 USC § 102(b)

Claims 1 and 2 stand rejected under 35 USC § 102(b) as anticipated by Narumi et al. (hereinafter "Narumi"). This rejection is traversed.

Claim 2 has hereby been cancelled, thereby making moot this portion of the rejection.

Claim 1 has hereby been amended to recite that the 67 kDa laminin receptor that is used to test compounds is expressed from a gene expression vector. Support for this amendment is found in the specification as filed, for example, in paragraph 0106 of the published application, where cloning into commercially available vectors that enable protein expression is discussed. Further references to expression vectors are found in paragraphs 0131, 0136, 0138, 0146, 0148, 0149, 0151 and 0152. Thus, this amendment does not include any new matter.

In contrast, Narumi teaches exposing cells from the human sarcoma cell line H1080 to an antibody against a 37kDa partial peptide fusion protein. 67kDa LR from these cells is not expressed from a gene expression vector. Therefore, Narumi does not anticipate the claimed subject matter of claims 1 and 2.

In addition, the present claims are directed to screening methods, whereas Narumi does not show or describe a screening method.

In view of the foregoing, Applicant respectfully requests reconsideration and withdrawal of this rejection.

New Claims

New claim 35 recites that the 67 kDa laminin receptor that is used in the screening method is in a form selected from the group consisting of a purified protein, a soluble protein, a protein bonded to a carrier, a protein fused with another protein, and a partial peptide of 37 kDa that has the ability to bind to laminin. Support for this new claim is found in paragraph [0107] of

the published application. Applicant notes that none of these forms of 67 kDa were utilized by Narumi, and thus the subject matter of new claim 36 is distinct from that of Narumi.

New claim 36 recites a method of screening a catechin for its ability to bind to 67 kDa, as indicative of its ability to cause cell growth-inhibiting or a cancer cell metastasis inhibiting effects. Applicant submits that this new claim does not add any new matter, since one variant of original claim 1 was that the test compound that was screened was a catechin. Applicant notes that Narumi does not teach any connection between 67 kDa and catechins and thus the subject matter of new claim 36 is distinct from that of Narumi.

New claims 37 and 38 recite the methods of claims 12 and 13, respectively, and recite the case in which the compound is an antibody. These claims do not add any new matter, their subject matter being taken directly from the independent claims on which they depend.

Applicant respectfully request consideration and allowance of new claims 35-38.

Other matters

Claims 1, 12 and 13 have hereby been amended to incorporate the subject matter or portions of the subject matter of cancelled claims 2, 14-16 and 31-33, to accord with the species that were elected in response to the Restriction Requirement.

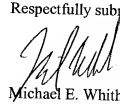
Concluding Remarks

In view of the foregoing, it is requested that the application be reconsidered, that claims 1, 12, 13, 16-17, and 33-38 be allowed, and that the application be passed to issue.

Should the Examiner find the application to be other than in condition for allowance, the Examiner is requested to contact the undersigned at 703-787-9400 (fax: 703-787-7557; email: mike@wcc-ip.com) to discuss any other changes deemed necessary in a telephonic or personal interview.

If an extension of time is required for this response to be considered as being timely filed, a conditional petition is hereby made for such extension of time. Please charge any deficiencies in fees and credit any overpayment of fees to Attorney's Deposit Account No. 50-2041.

Respectfully submitted,



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Green Tea Polyphenol Epigallocatechin-3-gallate Signaling Pathway through 67-kDa Laminin Receptor*[S]

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(−)-Epigallocatechin-3-gallate (EGCG), the principal polyphenol in green tea, has been shown to be a potent chemopreventive agent. Recently, 67-kDa laminin receptor (67LR) has been identified as a cell surface receptor for EGCG that mediates the anticancer activity of EGCG. Indeed, expression of 67LR confers EGCG responsiveness to tumor cells; however, the molecular basis for the anticancer activity of EGCG *in vivo* is not entirely understood. Here we show that (i) using a direct genetic screen, eukaryotic translation elongation factor 1A (eEF1A) is identified as a component responsible for the anticancer activity of EGCG; (ii) through both eEF1A and 67LR, EGCG induces the dephosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr-696 and activates myosin phosphatase; and (iii) silencing of 67LR, eEF1A, or MYPT1 in tumor cells results in abrogation of EGCG-induced tumor growth inhibition *in vivo*. Additionally, we found that eEF1A is up-regulated by EGCG through 67LR. Overall, these findings implicate both eEF1A and MYPT1 in EGCG signaling for cancer prevention through 67LR.

Many of the current anticancer drugs are natural products or derivatives thereof, illustrating the utility of natural products in drug discovery (1, 2). Green tea has been shown to have cancer-preventive activity in a variety of organ sites in animal models (3–5) and humans (6). Among the green tea constituents, (−)-epigallocatechin-3-gallate (EGCG)[2] is the most abundant and most active constituent in inhibiting experimental carcinogenesis and related reactions. Although many mechanisms for the anticancer activities of EGCG have been proposed based mainly on studies in cell lines (4, 7), it is still not clear which EGCG-induced molecular events are responsible for its cancer-preventive activity *in vivo*. Recently, we have identified 67-kDa laminin receptor (67LR) as a cell surface EGCG receptor that mediates the anticancer action of EGCG (8), and others showed that RNAi-mediated silencing of 67LR results in abrogation of EGCG-induced apoptosis in myeloma cells (9).

67LR is a nonintegrin laminin receptor and known to be overexpressed on the cell surface of various tumor cells (10). The expression level of this protein strongly correlates with the risk of tumor invasion and metastasis (10–12). Thus, it was postulated that 67LR plays a significant role in the tumor progression and speculated that studies conducted to define the function of 67LR could provide a new approach to cancer prevention. In this study, we tried to illuminate the cell signaling pathway mediated after the binding of EGCG to 67LR and its biologic and physiologic significance for the cancer-preventive activity of EGCG *in vivo*.

In an attempt to elucidate the pathways involved in the anticancer action of EGCG, we applied genetic suppressor element (GSE) methodology. GSEs are short cDNA fragments encoding peptides acting as dominant inhibitors of protein function or antisense RNAs inhibiting gene expression (13). GSEs behave as dominant selectable markers for the phenotype associated with the repression of the gene from which they derived, thus allowing identification of this gene. For example, this strategy previously allowed the demonstration that kinesin heavy chain is involved in the control of cell response to various DNA-damaging agents (14). To identify genes mediating cell sensitivity to EGCG, we selected GSEs conferring resistance to EGCG. Among genetic elements protecting cells from EGCG-induced cell growth inhibition, we isolated a GSE that encoded the N terminus of eukaryotic translation elongation factor 1A (eEF1A). eEF1A is an important component of the eukaryotic translation apparatus and is also known as a multifunctional protein that is involved in a large number of cellular processes (15). Here we show that eEF1A is indispensable for mediating anticancer activity of EGCG, and its protein expression level is up-regulated by EGCG through 67LR.

We previously reported that EGCG induces reduction of the phosphorylation of myosin regulatory light chain (MRLC) at Thr-18/Ser-19, and 67LR is essential for the activity of EGCG (16, 17). MRLC phosphorylation controls the activity of myosin II, a major motor protein in animal cells, which is involved in a wide range of processes, including muscle contraction, cell locomotion, cell division, and receptor capping (18). The phosphorylation of MRLC is regulated by two classes of enzymes: MLC kinases and myosin phosphatase (19). Myosin light chain kinase and Rho-kinase seem to be the two major kinases that

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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[2] The abbreviations used are: EGCG, (−)-epigallocatechin-3-gallate; 67LR, 67-kDa laminin receptor; eEF1A, eukaryotic translation elongation factor 1A; MYPT1, myosin phosphatase targeting subunit 1; MRLC, myosin regulatory light chain; GSE, genetic suppressor element; RNAi, RNA interference; FBS, fetal bovine serum; BSA, bovine serum albumin; shRNA, short hairpin RNA.

phosphorylate MRLC *in vitro* as well as *in vivo* (19). Myosin phosphatase is composed with three subunits: a 37-kDa catalytic subunit, a 20-kDa subunit of unknown function, and a 110 –130-kDa myosin phosphatase-targeting subunit (MYPT1) (20). The activity of myosin phosphatase is known to be regulated by phosphorylation of MYPT1, and two major sites, Thr-696 and Thr-853, have been extensively investigated and identified as an inhibitory site (20). In this study, we found that EGCG induces reduction of the MYPT1 phosphorylation at Thr-696, resulting in activation of myosin phosphatase, and both 67LR and eEF1A are responsible for the activity of EGCG. Moreover, we show that each of 67LR, eEF1A, and MYPT1 is indispensable for mediating EGCG signaling for cancer prevention *in vivo*. These results suggest that both eEF1A and MYPT1 are implicated in the EGCG signaling pathway through 67LR.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—EGCG, catalase, laminin, and anti-β-actin antibody were purchased from Sigma. Y-27632 was obtained from Calbiochem. Anti-67LR (F-18), anti-phospho-MRLC (Thr-18/Ser-19), anti-MLC2 (FL-172), anti-eEF1A (CBP-KK1), and anti-MYPT1 (H-130) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-MYPT1 (Thr-696) and anti-phospho-MYPT1 (Thr-853) antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture and DNA Transfection—B16 (a mouse melanoma), HepG2 (a human hepatocellular carcinoma), MCF-7 (a human breast carcinoma), HeLa (a human cervical carcinoma), and A431 (a human epidermoid carcinoma) were maintained in Dulbecco's modified Eagle's medium containing 5% (for B16 cells) or 10% (for the other cells) FBS. To assess cell proliferation, cells were treated with EGCG at the indicated concentrations for the indicated time periods in Dulbecco's modified Eagle's medium supplemented with 1% (for B16 cells) or 2% (for the other cells) FBS, 5 mg/ml BSA, and 200 units/ml catalase. FuGene6 transfection reagent (Roche Applied Science) was used for transient and stable transfection of cells, according to the manufacturer's protocol. For selecting stable clones, transfected cells were grown in medium containing G418 for neomycin resistance or hygromycin B for hygromycin resistance.

Preparation of a GSE cDNA Library—A GSE cDNA library was prepared from mouse embryo cDNA library (ML8000BB) (Clontech). The adaptors that contain ATG translation initiation codon-EcoRI/SphI site-TAG stop codon or SphI/EcoRI site were introduced into BglII/ClaI-digested pLPCX retroviral vector (Clontech), and EcoRI/SphI-digested mouse embryo cDNAs were subcloned into the modified pLPCX vectors. The ligation products were transformed into XL-10-blue, and a portion of the transformation was serially diluted and plated to estimate library complexity. The final GSE cDNA library had a complexity of 1×10^6 independent colonies or greater, with over 83% containing an insert.

Library Transduction and EGCG Selection—EcoPack2-293 cells and AmphoPack-293 cells (Clontech) were transfected with library DNA using FuGene6. The retrovirus-containing culture medium was collected 24, 36, 48, and 60 h later and filtered through a 0.45-μm filter. Target B16 cells were incubated with viral supernatant supplemented with 8 μg/ml Polybrene (Sigma) and 5% FBS. Infection was repeated four times at 12-h intervals. Then the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 50 μM EGCG, 1% FBS, 5 mg/ml BSA, and 200 units/ml catalase and fed every fourth day with the same medium for 3 weeks. Colonies were picked that arose at the concentration of EGCG that markedly reduced the growth of noninfected B16 cells.

Total RNA from 10^7 cells of each clone was reverse transcribed with Moloney mouse leukemia virus-reverse transcriptase (Amersham Biosciences) using oligo(dT)_{20} primer. The product was then amplified by PCR using Ex-Taq polymerase (Takara, Kyoto, Japan) with primers flanking the multicloning site of pLPCX, pLPCX-S (5′-GATCCGCTAGCGCTACCGGACTCAGAT-3′) and pLPCX-A (5′-CTTTCATTCCCCCCTTTTTCTGGAGAC-3′). PCR fragments were subcloned into the expression vector pTARGET (Promega, Madison, WI) and identified by DNA sequencing, using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster city, CA). For identification of GSEs conferring EGCG resistance, B16 cells transfected with each of the pTARGET vectors subcloned with individual PCR-amplified cDNAs were treated with EGCG in Dulbecco's modified Eagle's medium supplemented with 1% FBS, 5 mg/ml BSA, and 200 units/ml catalase for 96 h.

RNA Interference by Short Hairpin RNA (shRNA)—Target sequences for short hairpin RNAs for 67LR, eEF1A, and MYPT1 are as follows: shRNA for human 67LR, 5′-GGAGGAATTTCAGGGTGAA-3′; shRNA for mouse 67LR, 5′-CCTTCACTAACCAGATCCA-3′; shRNA for human and mouse eEF1A, 5′-TGCACCATGAAGCTTTGAG-3′; shRNA for human and mouse MYPT1, 5′-CTGTTTGTAAATGCTGCAGC-3′; shRNA for control, 5′-GCATATGTGCGTACCTAGCAT-3′. The annealed shRNA inserts were cloned into the psiRNA-hH1neo shRNA expression vector (for 67LR shRNA) or the psiRNA-hH1hygro shRNA expression vector (for eEF1A and MYPT1 shRNA) (InvivoGen, San Diego, CA) according to the manufacturer's protocol.

Western Blot Analysis—After stimulation, the cells were lysed in cell lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, and 1 mM pervanadate. Proteins were resolved on SDS-polyacrylamide gels and then transferred onto a nitrocellulose membrane. The membranes were blocked in 2.5% BSA and incubated with the antibody, followed by incubation with a secondary antibody. Proteins were visualized by using the ECL Advance kit (Amersham Biosciences). Band intensities were quantified using NIH Image-J software.

F-actin Staining—After stimulation, cells on the glass slide were fixed for 10 min in 3.7% formaldehyde at room temperature. These fixed cells were made permeable by incubating with 0.1% Triton X-100 for 5 min and then washed with phosphate-buffered saline. After blocking with 1% BSA, Alexa Fluor 488 phalloidin (Molecular Probes, Inc., Eugene, OR) was applied directly onto the glass slide and incubated for 1 h at 37 °C. After washing in phosphate-buffered saline, coverslips were mounted onto a glass side with a drop of mounting medium. Imaging was performed with a Nikon E600 microscope (Nikon,

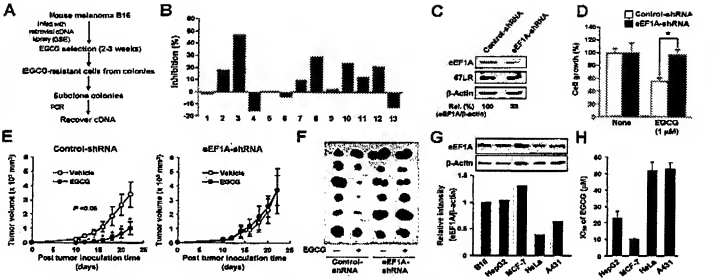

FIGURE 1. **Identification of eEF1A as a component responsible for cancer-preventive activity of EGCG.** *A*, *a flow chart* describing a genetic screen for regulators of EGCG-induced cell growth inhibition. *B*, B16 cells transfected with each isolated GSE sequence expression vector were treated with 50 μM EGCG for 96 h, and the cell numbers were assessed. The percentage inhibition of EGCG activity was calculated in reference to the activity of EGCG in the cells transfected with GSE empty vector and are the means (n = 2). *C*, eEF1A knockdown in B16 cells stably transfected with the eEF1A shRNA expression vector was confirmed by Western blot analysis. *D*, effect of eEF1A knockdown on EGCG-induced cell growth inhibition in B16 cells. Cells were treated with 1 μM EGCG for 96 h, and cell number was assessed. The results are shown as relative cell number to untreated control, and the data presented are the means ± S.D. (n = 3) (*, $p < 0.01$). *E*, C57BL/6N mice were subcutaneously inoculated with B16 cells stably transfected with the control shRNA or the eEF1A shRNA expression vector. Peroral administration of 0.1% EGCG was started 1 day before the cell inoculation. Tumor sizes are represented as the mean ± S.E. of six or seven mice. *F*, we excised tumors from mice 23 days after cell inoculation and photographed them. *G*, Western blot analysis of eEF1A in each cell line shown. *H*, the cell lines were treated for 96 h in the presence of EGCG. Cell number was calculated, and the IC_{50} value is expressed as the mean ± S.D. (n = 3).

Kanagawa, Japan). All images were acquired with a digital CCD camera (Nikon Digital Sight DS-5M-L1) and processed with custom software.

Tumor Growth in Vivo—B16 cells were detached and resuspended in phosphate-buffered saline. 5×10^5 cells in a single cell suspension were injected subcutaneously into the back of C57BL/6N mice (Charles River Laboratories Japan, Yokohama, Japan). They were kept at the Biotron Institute of Kyushu University in a 12-h light/12-h dark cycle (light on at 8 a.m.) in an air-conditioned room (20 °C and 60% humidity under specific pathogen-free conditions). EGCG was dissolved in vehicle (0.01% ascorbic acid solutions (pH 5.5) adjusted by NaOH). One day before inoculation, drinking water bottles were replaced by 0.1% EGCG or vehicle solutions, and administered solutions were replaced every other day. Tumor sizes were determined every other day via caliper measurements. The tumor volume was measured in two dimensions and calculated as follows: length/2 × width^2. Each data point represents the mean ± S.E. of tumor volumes from 6–7 animals. This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the law (number 105) and notification (number 6) of the Japanese government.

Statistical Analysis—Data for tumor growth *in vivo* were analyzed by a Mann-Whitney U test. The other data were analyzed by Student's t test. A level of $p < 0.05$ was considered significant.

RESULTS

Identification of eEF1A as a Component Responsible for Anticancer Activity of EGCG—To search for the mediators of EGCG-induced cell growth inhibition in B16 mouse melanoma cells, we utilized a targeted genetic screen with a GSE complementary DNA library, which was prepared from a mouse embryo (Fig. 1*A*). Under certain cell culture conditions, EGCG has been shown to produce hydrogen peroxide, which may exert various biological effects on cells (21). To distinguish between the direct effects of EGCG- and hydrogen peroxide-mediated effects, we examined whether catalase altered the inhibitory effect of EGCG on the cell growth of B16 cells. However, 50–800 units/ml catalase only partially inhibited the EGCG-induced cell growth inhibition; catalase did not completely prevent the effect of EGCG (supplemental Fig. 1). These results suggest that EGCG-induced cell growth inhibition in B16 cells mainly results not from hydrogen peroxide but from EGCG itself. We expected that the inactivation of genes essential for EGCG-induced cell growth inhibition by GSE sequences would allow cells to escape growth inhibition, continue cell growth, and eventually form cell colonies even in the presence of EGCG. By using this screen, we isolated multiple GSE sequences and individually tested their ability to confer EGCG resistance to B16 cells. As shown in Fig. 1*B*, the GSE numbered 3 was the most effective at evading EGCG-induced cell growth inhibition. The sequence for this GSE corresponded to the N terminus of eEF1A, a multifunctional protein that was originally identified as a cofactor for polypeptide elongation (15).

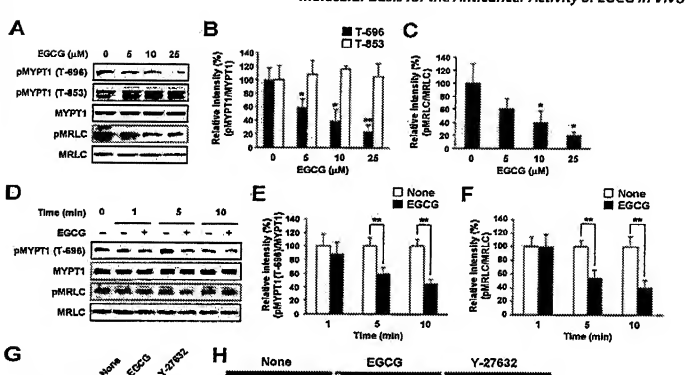

FIGURE 2. EGCG reduces the phosphorylation of MYPT1 at Thr-696 and induces actin cytoskeleton remodeling. *A*, MYPT1 phosphorylation (Thr-696 and Thr-853) and MRLC phosphorylation (Thr-18/Ser-19) in HeLa cells exposed to the indicated concentrations of EGCG for 10 min were analyzed by Western blot analysis. *B* and *C*, phosphorylation levels of MYPT1 at Thr-696 (*filled square*) or Thr-853 (*open square*) (*B*) and MRLC (*C*) were normalized to MYPT1 and MRLC, respectively. Band intensities were quantified using NIH Image J software. The results are shown as relative intensity to untreated control, and the data presented are the means ± S.D. ($n = 3$) (*, $p < 0.05$; **, $p < 0.01$ *versus* untreated control). *D*, MYPT1 phosphorylation (Thr-696) and MRLC phosphorylation (Thr-18/Ser-19) in HeLa cells exposed to 10 μM EGCG for the indicated time periods were analyzed by Western blot analysis. *E* and *F*, phosphorylation levels of MYPT1 (Thr-696) (*E*) and MRLC (*F*) were normalized to MYPT1 and MRLC, respectively. The results are shown as relative intensity to untreated control, and the data presented are the means ± S.D. ($n = 3$) (**, $p < 0.01$ *versus* untreated control). *G* and *H*, HeLa cells exposed to 10 μM EGCG or 10 μM Y-27632 for 30 min were analyzed by Western blot analysis (*G*) and stained with phalloidin (*H*).

To investigate the role of eEF1A in EGCG-induced cell growth inhibition, we used stable RNAi (22) to silence eEF1A expression in B16 cells (Fig. 1*C*). Remarkably, silencing of eEF1A attenuated the inhibitory effect of 1 μM EGCG on cell growth (Fig. 1*D*). In contrast, overexpression of eEF1A enhanced the inhibitory effects of 1 μM EGCG on cell growth (supplemental Fig. 2). This concentration is similar to the amount of EGCG found in human plasma after drinking more than two or three cups of green tea (23). EGCG is the only known polyphenol present in plasma in large proportion (77–90%) in a free form, although the other catechins are highly conjugated with glucuronic acid and/or sulfate group (24). Based on these considerations, the activities observed at 1 μM EGCG are relevant to the *in vivo* situations. Given this, we investigated the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with eEF1A-ablated B16 cells. Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harboring a control shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with eEF1A-ablated B16 cells (Fig. 1, *E* and *F*), indicating that eEF1A is involved in EGCG-induced cancer prevention.

EGCG induces growth inhibition in many cell lines; however, the efficacy of inhibition varied, depending on the cell lines used (25). We hypothesized that the expression level of eEF1A in a cell line correlates to the efficacy of EGCG-induced cell growth inhibition in that cell line. We investigated the expression levels of eEF1A in B16 cells and the following human cancer cell lines: hepatoma HepG2, breast carcinoma MCF-7, cervical carcinoma HeLa, and squamous cell carcinoma A431. The levels of eEF1A expression in B16 cells, HepG2 cells, and MCF-7 cells were relatively higher than those in HeLa cells and A431 cells (Fig. 1*G*). EGCG appeared to display different efficacies of growth inhibition in these cell lines, with estimated IC_{50} values of 9.7 μM for MCF-7 cells, 22.7 μM for HepG2 cells, 51.6 μM for HeLa cells, and 52.8 μM for A431 cells, respectively (Fig. 1*H*). The expression level of eEF1A is elevated in cell lines that are more sensitive to the effect of EGCG. These results support our conclusion that eEF1A serves as a mediator for EGCG-induced cancer prevention.

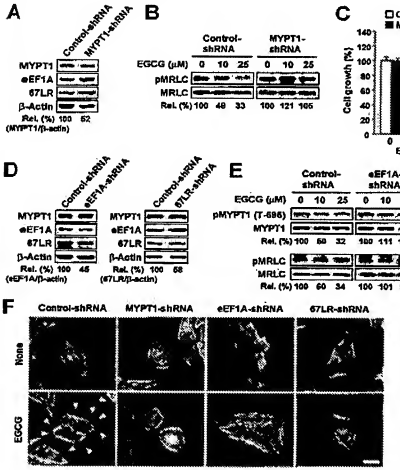

FIGURE 3. MYPT1 is involved in downstream EGCG signaling from both 67LR and eEF1A. *A* and *B*, Western blot analysis; *A*, MYPT1 knockdown in HeLa cells stably transfected with the MYPT1 shRNA expression vector. *B*, effect of MYPT1 knockdown on EGCG-induced reduction of the MRLC phosphorylation (Thr-18/Ser-19) level in HeLa cells. *C*, effect of MYPT1 knockdown on EGCG-induced cell growth inhibition in HeLa cells. The results are shown as indicated in Fig. 1*D* (*, $p < 0.001$). *D* and *E*, Western blot analysis; *D*, eEF1A and 67LR knockdown in HeLa cells stably transfected with the eEF1A shRNA expression vector and the 67LR shRNA expression vector, respectively. *E*, effect of knockdown of eEF1A or 67LR on EGCG-induced reduction of both MYPT1 phosphorylation (Thr-696) level and MRLC phosphorylation (Thr-18/Ser-19) level in HeLa cells. *F*, effect of knockdown of MYPT1, eEF1A, or 67LR on EGCG-induced actin cytoskeleton rearrangement in HeLa cells. Cells were treated with 10 μM EGCG for 10 min and stained with phalloidin. *Bar*, 30 μm.

EGCG Reduces the Phosphorylation of MYPT1 at Thr-696 and Induces a Dynamic Remodeling of Actin Cytoskeleton—Previously, we reported that EGCG-induced cell growth inhibition may result from the reduction of the phosphorylation of myosin regulatory light chain (MRLC) at Thr-18/Ser-19 through 67LR in HeLa cells (16). The activity of myosin phosphatase is known to be inhibited by phosphorylation of its targeting subunit MYPT1 at Thr-696 and Thr-853 (20). We tested the effect of EGCG on the phosphorylation of MYPT1 at Thr-696 and Thr-853. Intriguingly, although the phosphorylation level at Thr-853 was unaffected by EGCG, EGCG induced the dephosphorylation of MYPT1 at Thr-696 in a dose-dependent (Fig. 2, *A* and *B*) and time-dependent manner (Fig. 2, *D* and *E*). Further, this effect correlated with EGCG-induced reduction of the MRLC phosphorylation (Fig. 2, *A*, *C*, *D*, and *F*), suggesting that EGCG activates myosin phosphatase by reducing the MYPT1 phosphorylation level at Thr-696. In addition, we tested the effect of Y-27632, a specific inhibitor for Rho-kinase that phosphorylates MRLC (26, 27). Although Y-27632 induced the reduction of the MRLC phosphorylation similarly as EGCG, the MYPT1 phosphorylation level at Thr-696 was not reduced by Y-27632 (Fig. 2*G*).

It has been reported that MYPT1 directs the action of myosin phosphatase to not only MRLC but also other F-actin-binding proteins that influence cell contractility, morphology, and proliferation (28–30). We found that EGCG induces a dynamic remodeling of actin cytoskeleton. The cells treated with EGCG exhibited a filopodial-like structure (indicated by *arrowheads* in Fig. 2*H* and supplemental Fig. 3, *A* and *B*), and then after 3 h, the cell body retracted and left intracellular gaps (supplemental Fig. 3*C*), suggesting that the EGCG-induced filopodial-like projections are simple residual contact sites that have not yet been released from the substrate. We also found that cells treated with Y-27632 exhibited evident peripheral extensions that were clearly distinct from those of the cells treated with EGCG (indicated by *arrows*) (Fig. 2*H*). Together with the results in Fig. 2*G*, it is suggested that EGCG-induced actin cytoskeleton remodeling results from not only the reduction of the MRLC phosphorylation but also the activation of myosin phosphatase.

MYPT1 Is Involved in Downstream EGCG Signaling from both 67LR and eEF1A—Further, to establish whether MYPT1 is indeed involved in the suppressive effect of EGCG on MRLC phosphorylation and cell growth, we used stable RNAi to silence MYPT1 expression in HeLa cells. Western blot analysis indicated that stable RNAi for MYPT1 specifically silenced MYPT1 protein expression in HeLa cells with no effect on the expression of 67LR and eEF1A (Fig. 3*A*). Silencing of MYPT1 prevented both EGCG-induced reduction of the MRLC phosphorylation (Fig. 3*B*) and cell growth inhibition (Fig. 3*C*), suggesting that EGCG-induced dephosphorylation of MYPT1 at Thr-696 results in the activation of myosin phosphatase and inhibition of cell growth.

It has been reported that eEF1A binds to the ankyrin repeat of MYPT1 (31). It is tempting to speculate that both 67LR and eEF1A are upstream signaling components responsible for EGCG-induced dephosphorylation of MYPT1 at Thr-696. To test this hypothesis, we used stable RNAi to silence the expres-

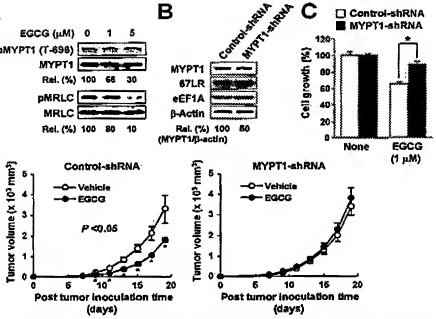

FIGURE 4. **MYPT1 is indispensable for cancer-preventive activity of EGCG *in vivo*.** *A*, MYPT1 phosphorylation (Thr-696) and MRLC phosphorylation (Thr-18/Ser-19) in B16 cells exposed to the indicated concentrations of EGCG for 20 min were analyzed by Western blot analysis. *B*, MYPT1 knockdown in B16 cells stably transfected with the MYPT1 shRNA expression vector was confirmed by Western blot analysis. *C*, effect of MYPT1 knockdown on 1 μM EGCG-induced cell growth inhibition in B16 cells. The results are shown as indicated in Fig. 1*D* (*, $p < 0.001$). *D*, C57BL/6N mice were subcutaneously inoculated with B16 cells stably transfected with the control shRNA or the MYPT1 shRNA expression vector. Peroral administration of 0.1% EGCG was started 1 day before the cell inoculation. Tumor sizes are represented as the mean ± S.E. of six or seven mice.

sion of 67LR or eEF1A in HeLa cells. We confirmed specific silencing of each target protein by stable RNAi in HeLa cells (Fig. 3*D*) and attenuation of the inhibitory effect of EGCG on cell growth in these cells (supplemental Fig. 4). In both 67LR-ablated HeLa cells and eEF1A-ablated HeLa cells, the inhibitory effect of EGCG on both the phosphorylation of MYPT1 at Thr-696 and the phosphorylation of MRLC was attenuated (Fig. 3*E*). In addition, EGCG-induced actin cytoskeleton rearrangement was no longer observed in MYPT1-, eEF1A-, or 67LR-ablated HeLa cells (Fig. 3*F*). These results suggest that MYPT1 is involved in downstream EGCG signaling from both 67LR and eEF1A.

MYPT1 Is Indispensable for Cancer-preventive Activity of EGCG in Vivo—Next, we investigated whether MYPT1 is involved in anticancer action of EGCG *in vivo*. In B16 cells, physiological concentrations of EGCG reduced the MYPT1 phosphorylation at Thr-696 and the MRLC phosphorylation as shown in Fig. 4*A*. We confirmed both silencing of MYPT1 by stable RNAi in B16 cells (Fig. 4*B*) and attenuation of the inhibitory effect of 1 μM EGCG on cell growth in MYPT1-ablated B16 cells *in vitro* (Fig. 4*C*). We tested the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with MYPT1-ablated B16 cells. Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harboring a control shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with MYPT-1-ablated B16 cells (Fig. 4*D*), suggesting that MYPT1 is indispensable for EGCG-induced cancer prevention.

Silencing of 67LR Blocks Cancer-preventive Activity of EGCG in Vivo—Although we have identified 67LR as a cell surface receptor for EGCG that mediates EGCG-induced cell growth inhibition (8), there is no validation of its implication in EGCG-induced cancer prevention *in vivo*. We investigated the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with 67LR-ablated B16 cells. We confirmed both silencing of 67LR by stable RNAi in B16 cells (Fig. 5*A*) and attenuation of the inhibitory effect of 1 μM EGCG on cell growth in 67LR-ablated B16 cells *in vitro* (Fig. 5*B*). Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harboring a control shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with 67LR-ablated B16 cells (Fig. 5, *C* and *D*), suggesting that 67LR functions as an EGCG receptor not only *in vitro* but also *in vivo*.

EGCG Up-regulates eEF1A Protein Expression through 67LR—Intriguingly, we found that the protein levels of eEF1A in tumor tissues from mice injected with control shRNA B16 cells were up-regulated by EGCG administration (Fig. 5*E*, *top*), whereas the eEF1A protein levels in tumor tissues from mice injected with 67LR-ablated B16 cells were not affected (Fig. 5*E*, *bottom*). eEF1A showed a subtle mobility shift on SDS-PAGE. Various post-translational modifications of eEF1A have been reported, including phosphorylation (31, 32), methylation (33), methylesterification (34), glutathionylation (35), and glucosylation (36). These post-translational modifications may affect the mobility of eEF1A on SDS-PAGE. We examined whether similar effects of EGCG treatment on eEF1A protein levels in tumor tissues could be recapitulated in EGCG-treated B16 cells *in vitro*. As shown in Fig. 5*F*, in control shRNA B16 cells, continued treatment with 1 μM EGCG for 6 days increased the eEF1A protein levels, whereas in 67LR-ablated B16 cells, such an effect of EGCG was not observed. These results indicate that eEF1A is up-regulated by EGCG, and 67LR is essential for the effect of EGCG on eEF1A.

Both 67LR and eEF1A Functions Upstream of MYPT1 in EGCG Signaling—The involvement of MYPT1 in downstream EGCG-triggered signaling from both 67LR and eEF1A was further documented by confirming abrogation of 1 μM EGCG-induced reduction of the MYPT1 phosphorylation level at Thr-696 and the MRLC phosphorylation in 67LR- or eEF1A-ablated B16 cells (Fig. 5*G*).

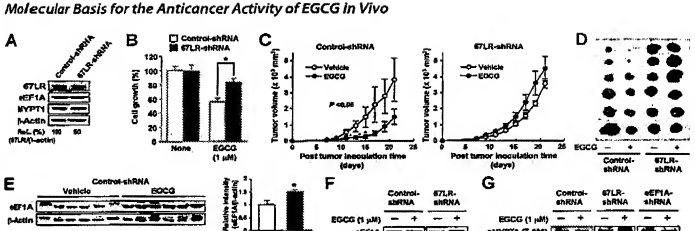

FIGURE 5. **67LR functions upstream of both eEF1A and MYPT1 in EGCG signaling for cancer prevention.** *A*, 67LR knockdown in B16 cells stably transfected with the 67LR shRNA expression vector was confirmed by Western blot analysis. *B*, effect of 67LR knockdown on 1 μM EGCG-induced cell growth inhibition in B16 cells. The results are shown as indicated in Fig. 1*D* (*, $p < 0.01$). *C*, C57BL/6N mice were subcutaneously inoculated with B16 cells stably transfected with the control shRNA or the 67LR shRNA expression vector. Peroral administration of 0.1% EGCG was started 1 day before the cell inoculation. Tumor sizes are represented as the mean ± S.E. of 6–7 mice. *D*, we excised tumors from mice 22 days after cell inoculation and photographed them. *E*, eEF1A expression in the excised tumor tissues. Western blot analyses of six tumor samples (six mouse samples) per group for eEF1A. *F*, effect of 67LR knockdown on 1 μM EGCG-induced up-regulation of eEF1A protein expression in B16 cells. Cells were treated with EGCG for 6 days, and culture medium with or without EGCG was refreshed everyday. *G*, effect of 67LR or eEF1A knockdown on 1 μM EGCG-induced reduction of both the MYPT1 phosphorylation (Thr-696) level and the MRLC phosphorylation (Thr-18/Ser-19) level in B16 cells. Cells were treated with EGCG for 20 min.

DISCUSSION

In this report, we have provided details on the molecular basis for the anticancer activity of EGCG both *in vitro* and *in vivo*. Through both the cell surface receptor 67LR and eEF1A, EGCG induces reduction of the MYPT1 phosphorylation at Thr-696, thus activating myosin phosphatase and inducing dephosphorylation of MRLC, as illustrated in Fig. 6. *In vivo*, EGCG-induced tumor growth inhibition was abrogated by silencing of 67LR, eEF1A, or MYPT1 in tumor cells, suggesting that the EGCG signaling mediated by 67LR, eEF1A, and MYPT1 is indispensable for the anticancer action of EGCG.

Selection of GSEs conferring resistance to a cytotoxic agent is a very powerful technique to identify drug sensitivity genes. In our search for GSEs protecting cells against EGCG-induced cell growth inhibition, we isolated a genetic element encoding the *N* terminus of eEF1A. eEF1A is an abundant G protein that delivers aminoacyl-tRNA to the elongating ribosome (15). Besides its canonical role in translation, eEF1A is known to be involved in several cellular processes, including embryogenesis, senescence, oncogenic transformation, and cell proliferation (37, 38). Here we show that eEF1A is responsible for mediating EGCG signaling for cancer prevention, and its protein expression is up-regulated by EGCG through 67LR. These results suggest that eEF1A functions downstream of 67LR and might act in a positive feedback loop to amplify the cascade of events following 67LR stimulation by EGCG, although the precise function of eEF1A within the EGCG signaling pathway remains to be defined. Accumulating evidence indicates that components of the translational apparatus have functions in cells beyond their conventional role in protein synthesis (39–41). Intriguingly,

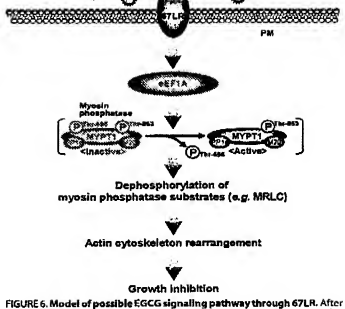

FIGURE 6. **Model of possible EGCG signaling pathway through 67LR.** After EGCG binding to 67LR, through eEF1A, the phosphorylation of MYPT1 at Thr-696 but not Thr-853 is reduced, which leads to the activation of myosin phosphatase. The activated myosin phosphatase dephosphorylates its substrates (e.g. MRLC), and actin cytoskeleton rearrangement is induced. The alteration of actin cytoskeleton might lead to cell growth inhibition.

67LR has been known to originate from the ribosomal protein p40, a component of the translational machinery (42). It is interesting that the extraribosomal functions of both 67LR and eEF1A conferred EGCG responsiveness to cells.

Rho-kinase is known to increase the MRLC phosphorylation not only by direct phosphorylation of MRLC but also by phosphorylating the MYPT1 at Thr-696 and Thr-853 and thereby inhibiting myosin phosphatase activity (20). Here we show that EGCG reduces both the MYPT1 phosphorylation at Thr-696 and the MRLC phosphorylation, whereas Y-27632, a specific inhibitor for Rho-kinase, reduces only the MRLC phosphorylation in HeLa cells. That the phosphorylation level of MYPT1 at Thr-696 is not responsive to Y-27632 is additionally observed in some cell lines (43, 44). Our observations establish that EGCG induces actin cytoskeleton reorganization that is clearly distinct from those of the cells treated with Y-27632. MYPT1-binding proteins have been found in addition to myosin (e.g. moesin and adducin). The localization of these proteins is regulated by their phosphorylation (28, 29), raising the possibility that the activation of myosin phosphatase via EGCG-induced MYPT1 dephosphorylation plays a role in EGCG-induced actin cytoskeleton reorganization. Intriguingly, it has been reported that MYPT1 binds to eEF1A (31), and more than half of the total eEF1A (>60%) binds to the actin cytoskeleton (45). Because other findings indicate that eEF1A is also implicated in microtubule binding, bundling, or severing (46, 47), a potential role for the protein in regulating cytoskeleton organization has also been proposed. Characterizing the mechanisms by which EGCG induces reduction of the MYPT1 phosphorylation at Thr-696 and reorganization of actin cytoskeleton through eEF1A should help in more precise understanding of cytoskeleton organization, although further experiments are necessary.

Our results provide new insights into the 67LR signaling pathway. Recently, some signaling pathways involving 67LR have been reported (48, 49). The receptor has been implicated in laminin-induced tumor cell attachment (50, 51), migration (52), and shear stress-dependent endothelial nitric-oxide synthase expression (53). However, laminin did not alter either cell growth or the level of the MRLC phosphorylation in HeLa cells (supplemental Fig. 5), suggesting that the 67LR-eEF1A-MYPT1 signaling pathway is peculiar to EGCG.

Chemoprevention by edible phytochemicals is now considered to be an inexpensive, readily applicable, acceptable, and accessible approach to cancer control and management (54); however, little is known about the mechanism of the chemopreventive action of most phytochemicals, including EGCG. Although previous studies have proposed various different mechanisms for cancer-preventive action of EGCG (4, 7, 54), it remains unclear which EGCG-induced molecular events are relevant *in vivo*. Here we show that each of 67LR, eEF1A, and MYPT1 is indispensable for EGCG-induced cancer prevention *in vivo*, and these proteins mediate physiological concentrations of EGCG-triggered unique signaling for cancer prevention. Our findings suggest that these proteins are "master proteins," which determine the efficacy of cancer-preventive activity of EGCG and have important implications for development and use of EGCG as a cancer-chemopreventive agent. Probably, only a tumor with a high expression level of these "master proteins" has sensitivity to physiological concentrations of EGCG. Our results not only illuminate the mechanisms for the cancer-preventive activity of EGCG but should help in the design of new strategies to prevent cancer and underscore the importance of tailoring cancer therapy on the basis of tumor genotype. Finally, in addition to cancer-chemopreventive properties, EGCG has been shown to possess diverse physiological activities, and we are curious to know whether EGCG signaling through the 67LR relates to other beneficial effects of EGCG.

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